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(71) Applicant *(for all designated States except US)*: AKZO N.V. [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : DIJKEMA, Rein [NL/NL]; Pensionarisstraat 6, NL-5345 ML Oss (NL). DE LEEUW, Renato [NL/NL]; Cesar Francklaan 16, NL-5343 EV Oss (NL).

(74) Agent: HERMANS, Franciscus, Guillielmus, Maria; Postbus 20, NL-5340 BH Oss (NL).

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(54) Title: HUMAN GONADOTROPIN RECEPTOR (FSH RECEPTOR)

(57) Abstract

The present invention provides a new receptor for gonadotropins, amino acid sequences of said receptor, nucleic acid sequences coding for said receptor, recombinant hosts comprising such a nucleic acid as well as screening assays using said receptor and ligands such as antibodies for that receptor. In a more preferred embodiment the invention provides antibodies raised against the human FSH receptor. The compounds and methods of the invention will find their use in the field of reproductive medicine, particularly human reproductive medicines.

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HUMAN GONADOTROPIN RECEPTOR (FSH RECEPTOR)

The invention relates to the field of reproductive medicine, particularly human reproductive medicine.

In that field frequent use is made of gonadotropins or analogs thereof. Gonadotropins (follicle stimulating hormone (FSH), chorionic gonadotropin (CG), luteinizing hormone (LH) and thyroid stimulating hormone (TSH)) are a family of protein hormones with a common α-subunit and a hormone specific β-subunit. All human gonadotropins have been studied intensively and much is known about them. Though there are clinical applications for the natural gonadotropins, either isolated from body fluids or produced by recombinant DNA technology, the natural gonadotropins often may not have the right combination of desired properties.

Therefore there is a need for altered gonadotropins, or molecules mimicking certain properties of gonadotropins. Especially interesting are derivatives with higher binding affinities for their respective receptors, either activating or blocking said receptors, derivatives with a longer residence time in the body or at the receptor and derivatives with a higher specificity for their receptors. This list of desirable alterations is of course not exhaustive.

However, in order to be able to study the effect of alterations made to the various gonadotropins, one must be able to study the interaction of the gonadotropin derivative with its receptor.

The usual means to study this interaction is studying the in vitro binding of the altered gonadotropin to a tissue or a cell line known to express the receptor involved. Usually such cell lines or tissues express more than one receptor. Especially when testing altered gonadotropins, the presence of other receptors may interfere with the test for binding affinity for the gonadotropin receptor, because the binding affinity and specificity are altered. Therefore the need exists for cells which only express one receptor, namely the one tested for.

The present invention provides such a cell. It also provides a novel gonadotropin receptor, i.e. the human follicle stimulating hormone receptor.

Both its DNA sequence (SEQ ID NO: 1) as well as its amino acid sequence (SEQ ID NO: 2) are provided. The DNA sequence is useful for studying the characteristics of the receptor by site directed mutations, thereby enabling to elucidate the parts of the receptor involved in the various aspects of its functions. The amino acid sequence can be used to produce synthetic peptides in order to identify the smallest peptide still having binding affinity for FSH.

In this way polypeptides comprising the extracellular part of the FSH receptor can be constructed. The peptides and polypeptides of the invention can also be used directly as competing compounds for the endogenous receptors or in diagnostic test kits testing for the ligand for the receptor.

Antibodies or antiserum directed against a polypeptide according to the invention have use in diagnostic immunoassay's and generation of anti-idiotype antibodies. A more preferred use is the use as an antidote against overstimulation with FSH. This occurs regularly in IVF (in vitro fertilization) protocols resulting in ovarian hyperstimulation. The antibodies may be formulated into pharmaceutical formulations by mixing with suitable pharmaceutical acceptable carriers in a manner known to those skilled in the art.

A specific polypeptide according to the invention in any of the embodiments described above can be used to produce antibodies, both polyclonal, monospecific and monoclonal. Such use of a polypeptide according to the invention and such an antibody fall within the scope of the invention.

When polyclonal antibodies are desired, techniques for producing and processing polyclonal sera are known in the art (e.g. Mayer and Walter, eds, Immunochemical Methods in Cell and Molecular Biology, Academic Press, London, 1987). In short, a selected mammal, e.g. a rabbit is given (multiple) injections with one of the above-mentioned immunogens, e.g. corresponding to about 20 μg to about 80 μg of polypeptide per immunization. Immunization is carried out with an acceptable adjuvant, generally in equal volumes of immunogen and adjuvant. Acceptable adjuvants include Freund's complete, Freund's incomplete, alum-precipitate or water-in-oil emulsions, with a preference for Freund's complete adjuvant for the initial immunization. For booster immunization Freund's incomplete adjuvant is preferred. The initial immunization consists of the administration of approximately 1 ml emulsion at multiple subcutaneous sites on the backs of the rabbits. Booster immunizations utilizing an equal

volume of immunogen are given at about one monthly intervals and are continued until adequate levels of antibodies are present in an individual rabbits serum. Blood is collected and serum isolated by methods known in the art.

Monospecific antibodies to each of the immunogens are affinity purified from polyspecific antisera by a modification of the method of Hall et al. (Nature 311, 379-387 1984), prepared by immunizing rabbits as described above with the purified proteins. Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogeneous binding characteristics for the relevant antigen. Homogeneous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope.

Monoclonal antibody reactive against one of the above-mentioned immunogens can be prepared by immunizing inbred mice, preferably Balb/c with the appropriate protein by techniques known in the art (Kohler and Milstein, Nature 256; 495-497, 1975). Hybridoma cells are subsequently selected by growth in hypoxanthine, thymidine and aminopterin in an appropriate cell culture medium such as Dulbecco's modified Eagle's medium (DMEM). Antibody producing hybridomas are cloned, preferably using the soft agar technique of MacPherson, (Soft Agar Techniques, Tissue Culture Methods and Applications, Kruse and Paterson, eds., Academic Press, 276, 1973). Discrete colonies are transferred into individual wells of culture plates for cultivation in an appropriate culture medium. Antibody producing cells are identified by screening with the appropriate immunogen. Immunogen positive hybridoma cells are maintained by techniques known in the art. Specific anti-monoclonal antibodies are produced by cultivating the hybridomas in vitro or preparing ascites fluid in mice following hybridoma injection by procedures known in the art. The

polyclonal or monoclonal antibodies can show agonistic or antagonistic activity as compared to the activity of the natural ligand. Specific antagonistic or agonistic activity may involve the interference of the antibodies with the signal transduction system in the cells expressing the FSH receptor, without competing for the binding of FSH to its receptor.

The DNA coding for the receptor or for an altered receptor can be inserted into a suitable vector for expression in either a prokaryotic or a eukaryotic host. The hosts may be bacteria, phages, yeasts, funghi, animal cells or plant cells, preferred however are mammalian cells.

The vector into which the DNA is inserted may be any suitable vector. It may comprise suitable regulating elements such as promoters, enhancers, repressors, etc. If necessary, it may also comprise a signal sequence to transport the protein translated from the DNA to the surface or the outside of the host, even though the receptor does contain its own signal sequence.

In order to make a test for binding activity of compounds for the receptors of the invention it will suffice to have a cell, which preferably expresses no related receptors and more preferably no other receptors at all, and which cell expresses the receptor according to the invention in altered or unaltered form, in its (outer) membrane. In order to test for activity in activating or blocking the receptor a signal producing system in the cell is needed. The signal producing system may be the cell's own or may be cotransfected with the DNA coding for the receptor.

Usually, the signal will be provided by the so called second messenger system, which works through G-proteins which are associated with the receptor at its surface site. Any other signal producing system as well as second messenger initiated test system will be suitable, as long as the signal is somehow measurable.

EXAMPLES

1. Molecular cloning of human follicle stimulating hormone receptor(hFSH-R)

1.1. Probe synthesis

Oligodeoxynucleotides were synthesized using the phosphoramidite method on an Applied Biosystems 381A DNA synthesizer. They correspond to nucleotide position 1237-1255 (transmembrane region II) and 1843-1861 (transmembrane region VII) of the hFSH-R (Minegish et al., Bioch.Bioph.Res.Comm. 175, 1125, 1991). Both oligonucleotides were used as primers in a polymerase chain reaction (Maniatis et al., Cold Spring Harbor Laboratory, "Molecular Cloning: A Laboratory Manual", 1989) to generate a 625bp DNA fragment amplified from human genomic DNA (Clonetech). The resulting product from PCR was verified by DNA sequence analysis (Pharmacia,T7-sequencing kit).

1.2. cDNA library

A human testis cDNA library in phage λgt11 (Clonetech; $1x10^6$ independent clones) was titrated on the host E.coli $Y1090^-$ and preparation of library DNA onto nitrocellulose filters was as described (Huynh et al., DNA Cloning Techniques, "A Practical Approach", 1984; Maniatis et al., ibid.).

1.3. Clone identification

The partial hFSH-R amplification product obtained by PCR (section 1.1.) was used as probe (Pharmacia, oligolabelling kit) to screen $3.6x10^5$ recombinant phages of the human testis cDNA library (section 1.2.). Prehybridization of filters was carried out for 5 hours at 65 °C in a solution containing 6xSSC (0.9 mol/l NaCl,0.09 mol/l Na-citrate, pH 7.0), 10xDenhardt (1xDenhardt:200µg/ml Ficoll-70 (Pharmacia), 200 µg/ml polyvinylpyrrolidone (Sigma), 200µg/ml bovine serum albumin (BSA, Sigma), 50 µg/ml sheared and denatured herring sperm DNA (Sigma), 9% dextran sulphate (Pharmacia), and 0.1% sodium dodecyl sulphate (SDS, Sigma). Hybridization was performed in the same solution by the addition of the ^{32}P-labelled DNA probe ($2.3x10^5$cpm/ml). The mixture was incubated overnight at the same temperature. Filters were washed in solutions with decreasing salt concentrations (0.1xSSC, 65 °C). Positive recombinant phages were purified by two successive rounds of phage titration and hybridization. Phage DNA inserts were isolated and subcloned in the endoR EcoRI site of pGEM3Z (Promega) and characterized by endoR mapping and DNA sequence analysis.

2. Expression of hFSH-R in chinese hamster ovary(CHO) cells

2.1. Construction of expression plasmids

A complete hFSH-R encoding cDNA was reconstructed from two overlapping cDNA clones (pGEM3ZR13 and pGEM3ZR25) by use of the unique internal endoR BamHI site at position 686 (Minegish et al., ibid.). The complete hFSH-R cDNA (pGEM3Zc1) is located on a 2222bp endoR EcoRI fragment (see Fig. 1). After isolation and filling in the endoR EcoRI sites with the Klenow fragment of DNA polymerase I (Pharmacia) the fragment was inserted into the unique endoR BamHI site (after filling in with Klenow fragment) of vector pKCR (O'Hara et al.,Proc.Natl.Acad.Sci.USA 78, 1527,1981). The latter vector was modified in a way that the last exon region of the β-globin gene was removed by digestion with endoR EcoRI and BamHI, filling in, and religation (position 1122-1196; van Ooyen et al., Science 206, 337, 1979) and replacement of pBR322 for pBR327 sequences.

2.2. Growth,transformation,and selection of CHO cells

CHO cells (CHO K1) were obtained from ATCC (CCL61). They were cultured in M505 medium that consisted of a mixture (1:1) of Dulbecco's Modified Eagle's Medium (DMEM, Gibco 074-2100) and Nutrient mixture F12 (Ham's F12, Gibco 074-1700) supplemented with 2.5 mg/ml sodium bicarbonate (Baker), 55 µg/ml sodium pyruvate (Fluka), 2.3 µg/ml β-mercaptoethanol (Baker), 1.2 µg/ml ethanolamine (Baker), 360 µg/ml L-glutamine (Merck), 0.45 µg/ml sodiumselenite (Fluka), 62.5 µg/ml penicillin (Mycopharm), 62.5 µg/ml streptomycin (Serva), and 10% fetal calf serum (FCS, Bocknek).

Recombinant constructs used for transformation of CHO cells consist of the expression vector pKCRhFSH-R (section 2.1.) and the selection vector pAG60MT2. The latter vector was constructed by insertion of a 3kb human $MTII_A$-containing endoR HindIII fragment (Karin and Richards, Nature 299, 797, 1982) into the endoR HindIII site of pAG60 (Colbère-Garapin et al., J.Mol.Biol 150, 1, 1981); the transcription of this gene was directed towards the tk-promoter that was located in front of the neomycin resistence gene.

For stable transformation the recombinant vectors pKCRhFSH-R and pAG60MT2 (molar ratio 10:1) were introduced in CHO cells by the calcium-phosphate precipitation method (Graham and van der Eb, Virology 52, 456, 1973). To select CHO transformants for stable integration and expression of the neomycin gene the antibiotic G418 (Gibco) was added 24 hours post-transformation at a concentration of 0.8 mg/ml. After this primary selection phase a second selection was performed by subjecting the cells to increasing concentrations of $CdCl_2$ as described (Greene et al., Mol.Endocrinology 4, 1465, 1990). In this way CHOhFSH-R1Cd10 refers to transformed CHO cells obtained after successive selection by neomycin and 10 µmol/l $CdCl_2$.

3. Characterisation of hFSH binding and signal transduction

3.1. Hormones

Highly purified (≥ 99%) lyophilized recombinant human FSH (recFSH batch 77; specific activity 10.661 (8.859 - 12.765) IU/mg in vitro bioactivity in terms of IS 70/45) was supplied by Diosynth (Oss, The Netherlands). Purified iodinated pituitary human FSH (^{125}I-hFSH; 3.3 - 7.4 MBq/µg) was obtained from New England Nuclear-Du Pont (NEN, Boston, MA, USA).

3.2. Scatchard analysis of FSH binding

CHOhFSH-R1Cd10 cells pellet were homogenised with a teflon glass homogeniser in ice-cold 10 mmol/l Tris-HCl buffer, pH 7.4, supplemented with 0.25 mol/l sucrose and 5 mmol/l $MgCl_2$. The homogenate was diluted to 2.5 x 10^6 cells (starting material)/ml with homogenisation buffer. For saturation experiments, aliquots homogenate (200 µl/tube) were incubated with increasing concentrations ^{125}I-hFSH (200 µl/tube; 4-150 pmol/l) with or without excess unlabeled recFSH (10 IU/100 µl/tube) in polypropylene microfuge tubes. The assay buffer consisted of 10 mmol/l Tris-HCl, pH 7.4, supplemented with 5 mmol/l $MgCl_2$ and 1 g/l bovine serum albumine (BSA; Sigma, St Louis, MO, USA). After 24 h of incubation at room temperature (RT), 500 µl ice-cold assay buffer was added, and bound and free hormone were separated by centrifugation (5 min at 15.000 x g). The bound radioactivity was measured with a LKB gamma counter. The K_d (equilibrium dissociation constant) and B_{max} (maximum binding capacity) were assessed by Scatchard plot analysis.

Competition of antisera for ^{125}I-FSH binding was assessed using the single cell clone mentioned previously expressing the human FSH-R, selected by subsequent exposure to G418 (800 µg/ml; Geneticin; Gibco) and Cadmium (2,5 µM). ^{125}I-FSH binding was performed essentially as described above, but the ligand (50,000 counts per minute) was mixed with varying dilutions of antisera prior to addition to the cell pellets. Binding of radioactive label is expressed as a percentage of maximal binding (%B/B_0). The control serum that is included is a serum of saline-injected mice. The results are presented in Figure 7.

3.3. FSH-induced cAMP production

The CHOhFSH-R1Cd10 cells were washed once, resuspended in M505, pH 7.4, supplemented with 10% FCS, and cultured in 24 well-plates (Nunclon; 0.1% gelatin coated) at a concentration of 2 x 10^5 cells/ml/well for 48 h at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2. After this preincubation period, cells were washed with M505 supplemented with 5 µg/ml transferrin (Pentex) and 1 µg/ml insulin (Diosynth) and incubated under the same conditions in 1 ml M505 supplemented with 5 µg/ml transferrin, 1 µg/ml insulin, 1 mM 3-isobutyl-1-methylxanthine (Aldrich-Europe, Beerse, Belgium) and various concentrations recFSH. After 10 min, 1, 4 and 22 h of incubation the supernatant medium was removed and stored at -20°C until cAMP analysis. To determine the intracellular cAMP content the remaining cells were treated with 0.5 ml 1-propanol which was followed by ultrasonification for 2 min. Subsequently, the content of each well was transfered to Eppendorf tubes and stored at -20°C. Prior to cAMP analysis using a cAMP (RIANEN) kit (NEN), the cellular samples were

lyophilized using a Speed Vac and reconstituted in 0.5 ml kit buffer. The medium samples were measured directly using a callibration curve of standard cAMP in CHO cell culture medium.

Interference of antisera with FSH-induced cAMP generation was assessed using the single cell clone mentioned previously expressing the human FSH-R, selected by subsequent exposure to G418 (800 µg/ml; Geneticin; Gibco) and Cadmium (2.5 µM). Second messenger generation experiments were performed essentially as described above, but the ligand (10 mU/ml recFSH) was mixed with varying dilutions of antisera prior to addition to the cells. The amount of extracellular cAMP generated was determined after a 24 hour incubation period. The control serum is one of saline-injected mice. The results are presented in Figure 8.

4. Raising of anti FSH-receptor antibodies.

4.1 Cloning of GST-FSH-receptor fusion protein constructs.

All recombinant DNA techniques were performed according to standard protocolls (Sambrook et al., in: Molecular Cloning, 2nd ed., CSHL Press). Three fusion proteins were generated, containing different parts of the extracellular region of the FSH-R, linked to glutathione-S-transferase (GST). Fusion protein 1 (GST-FSH-R 1) contains a 1000 basepair fragment coding for the complete extracellular N-terminus of the FSH-R. Fusion proteins 2 and 3 contain only parts of this sequence: fusion protein 2 (GST-FSH-R 2) contains a 600 basepair fragment coding for the N-terminal part of the FSH-R present in GST-FSH-R 1; fusion protein 3 (GST-FSH-R 3) contains a 400 basepair fragment coding for the C-terminal part of the FSH-R present in GST-

FSH-R 1). These fragments were derived by polymerase chain reaction (PCR) on the FSH-R cDNA clones (Figure 2A; seq. ID No. 1). Via this procedure restriction sites were introduced, which facilitated subsequent cloning procedures. For GST-FSH-R 1 we used the primers 5'-TGTCATCATCGGATC-3' and 5'-TCTGAGGATGTTGTAC-3'; for GST-FSH-R 2 we used the primers 5'-TGTCATCATCGGATC-3' and 5'-AGGCAGGGAATGGATCC-3'; for GST-FSH-R 3 we used the primers 5'-AGAACAAGGATCC-3' and 5'-TCTGAGGATGTTGTAC-3'. Some primers were complementary to the FSH-R cDNA sequence (SEQ ID NO:1). Only the complementary sequence of the primers is shown.

The FSH-R fragments were cloned into one of the pGEX expression vectors (Smith and Johnson, Gene 67, 31, 1988). Depending on the reading frame, either pGEX-1 (for the 1000 bp and 600 bp FSH-R fragments) or pGEX-3X (for the 400 bp FSH-R fragment) were used. In order to study expression of the fusion proteins, these constructs were transformed into E. coli MC1061.

4.2 Expression of GST-FSH-R fusion proteins in E. coli

Bacteria containing the GST-FSH-R fusion protein constructs were grown at 37°C to OD_{650} 1. To induce fusion protein expression isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a concentration of 0.1 mM, and cells were grown at 22°C for 4 hours. Cells were collected by centrifugation and resuspended in phosphate buffered saline (PBS) containing 1 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride (PMSF). After 20 minutes on ice, Triton-X-100 was added to a final concentration of 1%, incubation was continued for 10 minutes on ice, and cells were sonified. The supernantant of the sonicate was incubated with gluthathione-agarose carrier (Pharmacia) for 30

minutes at 4^0C. Carriers were washed several times with PBS, and finally with 50 mM Tris pH 8. The fusion proteins thus purified were used to generate antisera.

4.3 Immunisation of mice

Six weeks old female BALB/c mice were injected intraperitoneally with 50 µg of bacterial fusion proteins in complete Freund's adjuvant. In addition, mice were immunized with membranes of 10^7 CHO cells transfected with the human FSH-R. For this purpose an FSH-R single cell clone was used that was obtained by subsequent G418 (800 µg/ml; Geneticin, Gibco) and cadmium (2.5 µM) selection. Two subsequent intramuscular injections were given at three weeks intervals with 50 µg fusion protein or membranes of 10^7 cells in incomplete Freund's adjuvant. Three weeks after the third injection, mice were boosted intraperitoneally with 100 µg fusion protein or membranes of 10^7 cells in PBS. Four days after this final boost, sera and spleens were collected. Erythrocyte depleted spleen cells were prepared according to Steenbakkers et al. (J. Imm. Methods 152, 69, 1992).

4.4 Western immunoblotting

Denatured and reduced protein preparations were applied on sodium dodecylsulfate (SDS) polyacrylamide gels and blotted onto nitrocellulose filters. In order to be able to compare reactivity with different protein preparations, small (approximately 200 ng) and equivalent amounts of protein were applied to the gels. Membranes were blocked with 20% FCS, and incubated for 2 hours at room temperature with different concentrations of antibody preparations.

Blots were washed with Tris-buffered saline (TBS) - Tween and incubated with Goat-anti-mouse alkaline phosphatase (AP) conjugate (Promega) for 45 minutes at room temperature. Subsequently, AP substrate (nitroblue tetrazoline (NBT) / 5-bromo-4-chloro-3-indolyl phosphate (BCIP)) reactions were done in 100 mM NaCl, 100 mM Tris pH 9.5, and 10 mM MgCl2).

In order to show specific immunoreactivity with the FSH-R part of GST-FSH-R fusion proteins, antisera raised against bacterial fusion proteins were precleared with GST by pre-incubating sera several times with bacterially produced GST.

4.5 Immunostaining

CHO cells were attached to the bottom of 24 well plates (approximately 10^6 cells/well) for three days. Cells were washed in phosphate buffered saline (PBS) for 10 minutes. After fixation with 4% paraformaldehyde in PBS for one hour at room temperature, the cells were washed in PBS containing 0.05% normal swine serum (NSS) and 0.02% Triton-X-100 for 10 minutes. After blocking with NSS in PBS-Triton for 1 hour, cells were incubated with antisera for 16 hours at room temperature. After washing 3 times 10 minutes in PBS-NSS-Triton, cells were incubated with Swine-anti-mouse coupled to fluorescein isothiocyanate (FITC) for 1 hour at room temperature. Cells were washed in PBS and kept in mount solution to avoid fading of the fluorescent signal during examination with an inverted microscope.

Results

hFSH-R cDNA

Screening of the human testis cDNA library with the hFSH-R specific DNA probe resulted in five recombinant phages positive in hybridization. Two of these were studied in more detail and their insert size and map location are shown in figure 1.
In order to reconstruct a cDNA encoding the complete hFSH-R a combination was made between pGEM3ZR13 (endoR EcoRI-BamHI;position -84 to +686) and pGEM3ZR25(endoR BamHI-EcoRI; position +686 to +2138) giving pGEM3Zc1.

hFSH-R cDNA sequence

A complete DNA sequence analysis was performed of the 2222bp endoR EcoRI fragment of pGEM3Zc1 (Fig. 2A; Seq. ID. No. 1)). Comparison of this sequence with the hFSH-R cDNA sequence of Minegish et al. (ibid.) gave rise to several modifications of the hFSH-R protein. Differences between both sequences are shown in Figure 2 together with their corresponding change in amino acid.

Scatchard analysis of FSH binding

Scatchard plot analysis of the saturation data gave a straight line (Fig. 3, inset), indicating the presence of a single class of high affinity binding sites with a K_d = 28.8 pmol/l and B_{max} = 4.1 pmol/l, which equals approximately 2400 receptors per cell.

FSH-induced cAMP production

Incubation of CHOhFSH-R1Cd10 cells with increasing concentrations recFSH induced a dose-dependent increase in intracellular as well as extracellular (medium) cAMP at all incubation times. Intracellular cAMP reached its maximum at 1 h of incubation which was followed by a sharp decline reaching cAMP levels only slightly elevated compared to unstimulated conditions at 22 h of incubation (Fig. 4). Concomitantly with the decline in intracellular cAMP extracellular cAMP increased reaching the highest levels at 22 h of incubation (Fig. 5).

Characterization of antibodies against the human FSH-R

Antibodies raised against the human FSH-R were characterized by Western immunoblotting, immunostaining, and by their competition for ^{125}I-FSH binding and cAMP generation.

Antisera raised against the bacterial GST-FSH-R fusions recognized these proteins on Western blots (Fig. 6C). Often, these antisera also recognized GST alone (control lanes). After preclearing with GST, antisera specifically reacted with the FSH-R part of the fusion proteins. A representative example is shown in Figure 6B.

Immunostaining of prefixed CHO cells expressing the transfected human FSH-R showed a positive reaction with the antisera raised against bacterial fusion proteins, whereas nontransfected CHO cells showed no immunostaining.

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            125                 130                 135

GTT CAC AAG ATT CAT TCT CTC CAA AAA GTT TTA CTT GAC ATT CAA GAT   543
Val His Lys Ile His Ser Leu Gln Lys Val Leu Leu Asp Ile Gln Asp
        140                 145                 150

AAC ATA AAC ATC CAC ACA ATT GAA AGA AAT TCT TTC GTG GGG CTG AGC   591
Asn Ile Asn Ile His Thr Ile Glu Arg Asn Ser Phe Val Gly Leu Ser
    155                 160                 165

TTT GAA AGT GTG ATT CTA TGG CTG AAT AAG AAT GGG ATT CAA GAA ATA   639
Phe Glu Ser Val Ile Leu Trp Leu Asn Lys Asn Gly Ile Gln Glu Ile
170                 175                 180                 185

CAC AAC TGT GCA TTC AAT GGA ACC CAA CTA GAT GAG CTG AAT CTA AGC   687
His Asn Cys Ala Phe Asn Gly Thr Gln Leu Asp Glu Leu Asn Leu Ser
                190                 195                 200

GAT AAT AAT AAT TTA GAA GAA TTG CCT AAT GAT GTT TTC CAC GGA GCC   735
Asp Asn Asn Asn Leu Glu Glu Leu Pro Asn Asp Val Phe His Gly Ala
            205                 210                 215

TCT GGA CCA GTC ATT CTA GAT ATT TCA AGA ACA AGG ATC CAT TCC CTG   783
Ser Gly Pro Val Ile Leu Asp Ile Ser Arg Thr Arg Ile His Ser Leu
        220                 225                 230

CCT AGC TAT GGC TTA GAA AAT CTT AAG AAG CTG AGG GCC AGG TCG ACT   831
Pro Ser Tyr Gly Leu Glu Asn Leu Lys Lys Leu Arg Ala Arg Ser Thr
    235                 240                 245

TAC AAC TTA AAA AAG CTG CCT ACT CTG GAA AAG CTT GTC GCC CTC ATG   879
Tyr Asn Leu Lys Lys Leu Pro Thr Leu Glu Lys Leu Val Ala Leu Met
250                 255                 260                 265

GAA GCC AGC CTC ACC TAT CCC AGC CAT TGC TGT GCC TTT GCA AAC TGG   927
Glu Ala Ser Leu Thr Tyr Pro Ser His Cys Cys Ala Phe Ala Asn Trp
                270                 275                 280

AGA CGG CAA ATC TCT GAG CTT CAT CCA ATT TGC AAC AAA TCT ATT TTA   975
Arg Arg Gln Ile Ser Glu Leu His Pro Ile Cys Asn Lys Ser Ile Leu
            285                 290                 295
```

```
AGG CAA GAA GTT GAT TAT ATG ACT CAG ACT AGG GGT CAG AGA TCC TCT  1023
Arg Gln Glu Val Asp Tyr Met Thr Gln Thr Arg Gly Gln Arg Ser Ser
        300                 305                 310

CTG GCA GAA GAC AAT GAG TCC AGC TAC AGC AGA GGA TTT GAC ATG ACG  1071
Leu Ala Glu Asp Asn Glu Ser Ser Tyr Ser Arg Gly Phe Asp Met Thr
    315                 320                 325

TAC ACT GAG TTT GAC TAT GAC TTA TGC AAT GAA GTG GTT GAC GTG ACC  1119
Tyr Thr Glu Phe Asp Tyr Asp Leu Cys Asn Glu Val Val Asp Val Thr
330                 335                 340                 345

TGC TCC CCT AAG CCA GAT GCA TTC AAC CCA TGT GAA GAT ATC ATG GGG  1167
Cys Ser Pro Lys Pro Asp Ala Phe Asn Pro Cys Glu Asp Ile Met Gly
                350                 355                 360

TAC AAC ATC CTC AGA GTC CTG ATA TGG TTT ATC AGC ATC CTG GCC ATC  1215
Tyr Asn Ile Leu Arg Val Leu Ile Trp Phe Ile Ser Ile Leu Ala Ile
            365                 370                 375

ACT GGG AAC ATC ATA GTG CTA GTG ATC CTA ACT ACC AGC CAA TAT AAA  1263
Thr Gly Asn Ile Ile Val Leu Val Ile Leu Thr Thr Ser Gln Tyr Lys
        380                 385                 390

CTC ACA GTC CCC AGG TTC CTT ATG TGC AAC CTG GCC TTT GCT GAT CTC  1311
Leu Thr Val Pro Arg Phe Leu Met Cys Asn Leu Ala Phe Ala Asp Leu
    395                 400                 405

TGC ATT GGA ATC TAC CTG CTG CTC ATT GCA TCA GTT GAT ATC CAT ACC  1359
Cys Ile Gly Ile Tyr Leu Leu Leu Ile Ala Ser Val Asp Ile His Thr
410                 415                 420                 425

AAG AGC CAA TAT CAC AAC TAT GCC ATT GAC TGG CAA ACT GGG GCA GGC  1407
Lys Ser Gln Tyr His Asn Tyr Ala Ile Asp Trp Gln Thr Gly Ala Gly
                430                 435                 440

TGT GAT GCT GCT GGC TTT TTC ACT GTC TTT GCC AGT GAG CTG TCA GTC  1455
Cys Asp Ala Ala Gly Phe Phe Thr Val Phe Ala Ser Glu Leu Ser Val
            445                 450                 455

TAC ACT CTG ACA GCT ATC ACC TTG GAA AGA TGG CAT ACC ATC ACG CAT  1503
Tyr Thr Leu Thr Ala Ile Thr Leu Glu Arg Trp His Thr Ile Thr His
        460                 465                 470

GCC ATG CAG CTG GAC TGC AAG GTG CAG CTC CGC CAT GCT GCC AGT GTC  1551
Ala Met Gln Leu Asp Cys Lys Val Gln Leu Arg His Ala Ala Ser Val
    475                 480                 485

ATG GTG ATG GGC TGG ATT TTT GCT TTT GCA GCT GCC CTC TTT CCC ATC  1599
Met Val Met Gly Trp Ile Phe Ala Phe Ala Ala Ala Leu Phe Pro Ile
490                 495                 500                 505

TTT GGC ATC AGC AGC TAC ATG AAG GTG AGC ATC TGC CTG CCC ATG GAT  1647
Phe Gly Ile Ser Ser Tyr Met Lys Val Ser Ile Cys Leu Pro Met Asp
                510                 515                 520
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ATT GAC AGC CCT TTG TCA CAG CTG TAT GTC ATG TCC CTC CTT GTG CTC  1695
Ile Asp Ser Pro Leu Ser Gln Leu Tyr Val Met Ser Leu Leu Val Leu
            525                 530                 535

AAT GTC CTG GCC TTT GTG GTC ATC TGT GGC TGC TAT ATC CAC ATC TAC  1743
Asn Val Leu Ala Phe Val Val Ile Cys Gly Cys Tyr Ile His Ile Tyr
        540                 545                 550

CTC ACA GTG CGG AAC CCC AAC ATC GTG TCC TCC TCT AGT GAC ACC AGG  1791
Leu Thr Val Arg Asn Pro Asn Ile Val Ser Ser Ser Ser Asp Thr Arg
    555                 560                 565

ATC GCC AAG CGC ATG GCC ATG CTC ATC TTC ACT GAC TTC CTC TGC ATG  1839
Ile Ala Lys Arg Met Ala Met Leu Ile Phe Thr Asp Phe Leu Cys Met
570                 575                 580                 585

GCA CCC ATT TCT TTC TTT GCC ATT TCT GCC TCC CTC AAG GTG CCC CTC  1887
Ala Pro Ile Ser Phe Phe Ala Ile Ser Ala Ser Leu Lys Val Pro Leu
                590                 595                 600

ATC ACT GTG TCC AAA GCA AAG ATT CTG CTG GTT CTG TTT CAC CCC ATC  1935
Ile Thr Val Ser Lys Ala Lys Ile Leu Leu Val Leu Phe His Pro Ile
            605                 610                 615

AAC TCC TGT GCC AAC CCC TTC CTC TAT GCC ATC TTT ACC AAA AAC TTT  1983
Asn Ser Cys Ala Asn Pro Phe Leu Tyr Ala Ile Phe Thr Lys Asn Phe
        620                 625                 630

CGC AGA GAT TTC TTC ATT CTG CTG AGC AAG TGT GGC TGC TAT GAA ATG  2031
Arg Arg Asp Phe Phe Ile Leu Leu Ser Lys Cys Gly Cys Tyr Glu Met
    635                 640                 645

CAA GCC CAA ATT TAT AGG ACA GAA ACT TCA TCC ACT GTC CAC AAC ACC  2079
Gln Ala Gln Ile Tyr Arg Thr Glu Thr Ser Ser Thr Val His Asn Thr
650                 655                 660                 665

CAT CCA AGG AAT GGC CAC TGC TCT TCA GCT CCC AGA GTC ACC AAT GGT  2127
His Pro Arg Asn Gly His Cys Ser Ser Ala Pro Arg Val Thr Asn Gly
                670                 675                 680

TCC ACT TAC ATA CTT GTC CCT CTA AGT CAT TTA GCC CAA AAC          2170
Ser Thr Tyr Ile Leu Val Pro Leu Ser His Leu Ala Gln Asn
            685                 690                 695

TAAAACACAAT GTGAAAATGT ATCTGAAAAA AAAAAAAAAA AAACCGGAAT TC       2222
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 695 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Leu Leu Leu Val Ser Leu Leu Ala Phe Leu Ser Leu Gly Ser
1 5 10 15

Gly Cys His His Arg Ile Cys His Cys Ser Asn Arg Val Phe Leu Cys
20 25 30

Gln Glu Ser Lys Val Thr Glu Ile Pro Ser Asp Leu Pro Arg Asn Ala
35 40 45

Ile Glu Leu Arg Phe Val Leu Thr Lys Leu Arg Val Ile Gln Lys Gly
50 55 60

Ala Phe Ser Gly Phe Gly Asp Leu Glu Lys Ile Glu Ile Ser Gln Asn
65 70 75 80

Asp Val Leu Glu Val Ile Glu Ala Asp Val Phe Ser Asn Leu Pro Lys
85 90 95

Leu His Glu Ile Arg Ile Glu Lys Ala Asn Asn Leu Leu Tyr Ile Asn
100 105 110

Pro Glu Ala Phe Gln Asn Leu Pro Asn Leu Gln Tyr Leu Leu Ile Ser
115 120 125

Asn Thr Gly Ile Lys His Leu Pro Asp Val His Lys Ile His Ser Leu
130 135 140

Gln Lys Val Leu Leu Asp Ile Gln Asp Asn Ile Asn Ile His Thr Ile
145 150 155 160

Glu Arg Asn Ser Phe Val Gly Leu Ser Phe Glu Ser Val Ile Leu Trp
165 170 175

Leu Asn Lys Asn Gly Ile Gln Glu Ile His Asn Cys Ala Phe Asn Gly
180 185 190

Thr Gln Leu Asp Glu Leu Asn Leu Ser Asp Asn Asn Asn Leu Glu Glu
195 200 205

Leu Pro Asn Asp Val Phe His Gly Ala Ser Gly Pro Val Ile Leu Asp
210 215 220

Ile Ser Arg Thr Arg Ile His Ser Leu Pro Ser Tyr Gly Leu Glu Asn
225 230 235 240

Leu Lys Lys Leu Arg Ala Arg Ser Thr Tyr Asn Leu Lys Lys Leu Pro
245 250 255

Thr Leu Glu Lys Leu Val Ala Leu Met Glu Ala Ser Leu Thr Tyr Pro
260 265 270

Ser His Cys Cys Ala Phe Ala Asn Trp Arg Arg Gln Ile Ser Glu Leu
275 280 285

His Pro Ile Cys Asn Lys Ser Ile Leu Arg Gln Glu Val Asp Tyr Met
290 295 300

Thr Gln Thr Arg Gly Gln Arg Ser Ser Leu Ala Glu Asp Asn Glu Ser
305 310 315 320

Ser Tyr Ser Arg Gly Phe Asp Met Thr Tyr Thr Glu Phe Asp Tyr Asp
325 330 335

Leu Cys Asn Glu Val Val Asp Val Thr Cys Ser Pro Lys Pro Asp Ala
340 345 350

Phe Asn Pro Cys Glu Asp Ile Met Gly Tyr Asn Ile Leu Arg Val Leu
355 360 365

Ile Trp Phe Ile Ser Ile Leu Ala Ile Thr Gly Asn Ile Ile Val Leu
370 375 380

Val Ile Leu Thr Thr Ser Gln Tyr Lys Leu Thr Val Pro Arg Phe Leu
385 390 395 400

Met Cys Asn Leu Ala Phe Ala Asp Leu Cys Ile Gly Ile Tyr Leu Leu
405 410 415

Leu Ile Ala Ser Val Asp Ile His Thr Lys Ser Gln Tyr His Asn Tyr
420 425 430

Ala Ile Asp Trp Gln Thr Gly Ala Gly Cys Asp Ala Ala Gly Phe Phe
435 440 445

Thr Val Phe Ala Ser Glu Leu Ser Val Tyr Thr Leu Thr Ala Ile Thr
450 455 460

Leu Glu Arg Trp His Thr Ile Thr His Ala Met Gln Leu Asp Cys Lys
465 470 475 480

Val Gln Leu Arg His Ala Ala Ser Val Met Val Met Gly Trp Ile Phe
485 490 495

Ala Phe Ala Ala Ala Leu Phe Pro Ile Phe Gly Ile Ser Ser Tyr Met
500 505 510

Lys Val Ser Ile Cys Leu Pro Met Asp Ile Asp Ser Pro Leu Ser Gln
515 520 525

Leu Tyr Val Met Ser Leu Leu Val Leu Asn Val Leu Ala Phe Val Val
530 535 540

Ile Cys Gly Cys Tyr Ile His Ile Tyr Leu Thr Val Arg Asn Pro Asn
545 550 555 560

Ile Val Ser Ser Ser Ser Asp Thr Arg Ile Ala Lys Arg Met Ala Met
565 570 575

Leu Ile Phe Thr Asp Phe Leu Cys Met Ala Pro Ile Ser Phe Phe Ala
580 585 590

Ile Ser Ala Ser Leu Lys Val Pro Leu Ile Thr Val Ser Lys Ala Lys
595 600 605

Ile Leu Leu Val Leu Phe His Pro Ile Asn Ser Cys Ala Asn Pro Phe
610 615 620

Leu Tyr Ala Ile Phe Thr Lys Asn Phe Arg Arg Asp Phe Phe Ile Leu
625 630 635 640

Leu Ser Lys Cys Gly Cys Tyr Glu Met Gln Ala Gln Ile Tyr Arg Thr
645 650 655

Glu Thr Ser Ser Thr Val His Asn Thr His Pro Arg Asn Gly His Cys
660 665 670

Ser Ser Ala Pro Arg Val Thr Asn Gly Ser Thr Tyr Ile Leu Val Pro
675 680 685

Leu Ser His Leu Ala Gln Asn
690 695

LEGENDS

Fig. 1. Physical maps of three hFSH-R cDNA clones and their position on their respective plasmids.

Fig. 2A. Nucleotide sequence of the hFSH-R cDNA cloned in pGEM3Zc1 (seq. ID No. 1).

Fig. 2B. Amino acid sequence of the hFSH-R coded for by the hFSH-R cDNA in pGEM3Zc1 (seq. ID No. 2).

Fig. 3. Binding of ^{125}I-hFSH to hFSH-R of a neomycin-CdCl2 (10 µmol/l) selected CHO pool. Cell membranes were incubated with increasing concentrations ^{125}I-hFSH in the absence or presence of excess unlabeled recFSH. The saturation curve of specifically bound ^{125}I-hFSH together with the derived Scatchard plot are shown. The values represent the mean of duplicate determinations. The calculated Kd and B_{max} are presented in the Scatchard plot (insert).

Fig. 4. Dose dependent stimulation of intracellular cAMP by recFSH. The stimulations were repeated at different times of incubation. Values represent the mean of duplicate determinations.

Fig. 5. Dose dependent stimulation of extracellular (medium) cAMP by recFSH. The stimulations were repeated at different times of incubation. Values represent the mean of duplicate determinations.

Fig. 6A. Western blot of GST-hFSH-R fusion proteins stained with Coomassie Blue.

Fig. 6B. Western blot of GST-hFSH-R fusion proteins stained with antiserum raised against GST-hFSH-R3 fusion protein. The antiserum has been preclaered with GST proteins.

Fig. 6C. Western blot of GST-hFSH-R fusion proteins stained with antiserum raised against GST-hFSH-R1 fusion protein.

Fig. 7. Interference of hFSH-R antisera with binding of ^{125}I-FSH to CHO cells expressing the human FSH receptor (hFSH-R).

Fig. 8. Interference of hFSH-R antisera with FSH-induced cAMP generation.

CLAIMS.

1) An antibody or an antigen-binding fragment thereof having specific binding activity for the human Follicle Stimulating Hormone receptor (FSH-R).

2) An antibody or fragment thereof according to claim 1, characterised in that said antibody is a monoclonal antibody.

3) An antibody or fragment thereof according to claim 1 or 2, characterized in that said antibody or fragment has agonistic or antagonistic activity with respect to the activity of FSH.

4) An anti idiotypic antibody or a functional fragment thereof capable of binding to an antibody or a fragment according to claims 1 or 2.

5) Pharmaceutical formulation comprising a therapeutical amount of an antibody or antigen-binding fragment according to any of the claims 1 to 4 and a pharmaceutical acceptable carrier.

6) A proteinaceous substance having binding activity for at least human follicle stimulating hormone, characterized in that the proteinaceous substance is the human Follicle Stimulating Hormone (FSH) receptor or an analogon or a derivative or a fragment thereof.

7) A proteinaceous substance according to claim 6, characterized in that it comprises a sequence at least 70% homologous to the sequence of SEQ ID NO:2 or a part thereof.

LEGENDS

Fig. 1. Physical maps of three hFSH-R cDNA clones and their position on their respective plasmids.

Fig. 2A. Nucleotide sequence of the hFSH-R cDNA cloned in pGEM3Zc1 (seq. ID No. 1).

Fig. 2B. Amino acid sequence of the hFSH-R coded for by the hFSH-R cDNA in pGEM3Zc1 (seq. ID No. 2).

Fig. 3. Binding of ^{125}I-hFSH to hFSH-R of a neomycin-CdCl2 (10 µmol/l) selected CHO pool. Cell membranes were incubated with increasing concentrations ^{125}I-hFSH in the absence or presence of excess unlabeled recFSH. The saturation curve of specifically bound ^{125}I-hFSH together with the derived Scatchard plot are shown. The values represent the mean of duplicate determinations. The calculated Kd and B_{max} are presented in the Scatchard plot (insert), where B/F is plotted against the specific bound.

Fig. 4. Dose dependent stimulation of intracellular cAMP by recFSH. The stimulations were repeated at different times of incubation. Values represent the mean of duplicate determinations.

Fig. 5. Dose dependent stimulation of extracellular (medium) cAMP by recFSH. The stimulations were repeated at different times of incubation. Values represent the mean of duplicate determinations.

Fig. 6A. Western blot of GST-hFSH-R fusion proteins stained with Coomassie Blue.

induces a response in the signal producing system of the transfected cell.

17) A transfected cell according to claim 16, characterized in that the signal producing system gives a measurable signal.

18) A method for testing compounds for human gonadotropin like activity, characterized in that the compound is contacted with a transfected cell according to claim 17 and that the signal of the signal producing system is detected or measured.

19) A method for testing compounds for antagonistic activity of human gonadotropin like activity, characterized in that said compound is contacted with a transfected cell according to claim 17 together with a compound with known human gonadotropin like activity and that the presence or absence of a signal of the signal producing system is detected or measured.

20) A method according to claim 18 or 19, characterized in that the human gonadotropin like activity is human follicle stimulating hormone activity.

Figure 1.

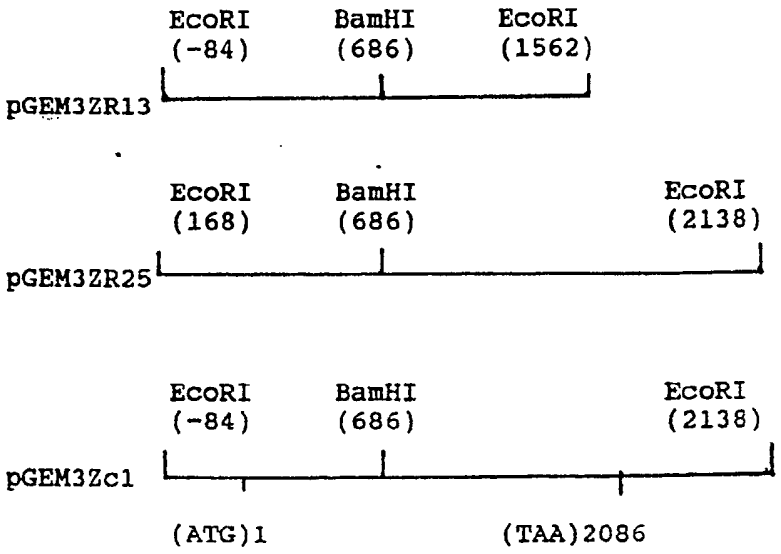

Figure 2.

		Minegish	pGEM3Zc1	Change
position	335	ACC	AAC	Thr to Asn
position	438	AAG	AAA	Silent(Lys)
position	590	GCA	GAG	Ala to Glu
position	592	GTG	CTG	Val to Leu
position	919	GCT	ACT	Ala to Thr
position	1731	CTG	CTC	Silent(Leu)
position	2039	AGT	AAT	Ser to Asn

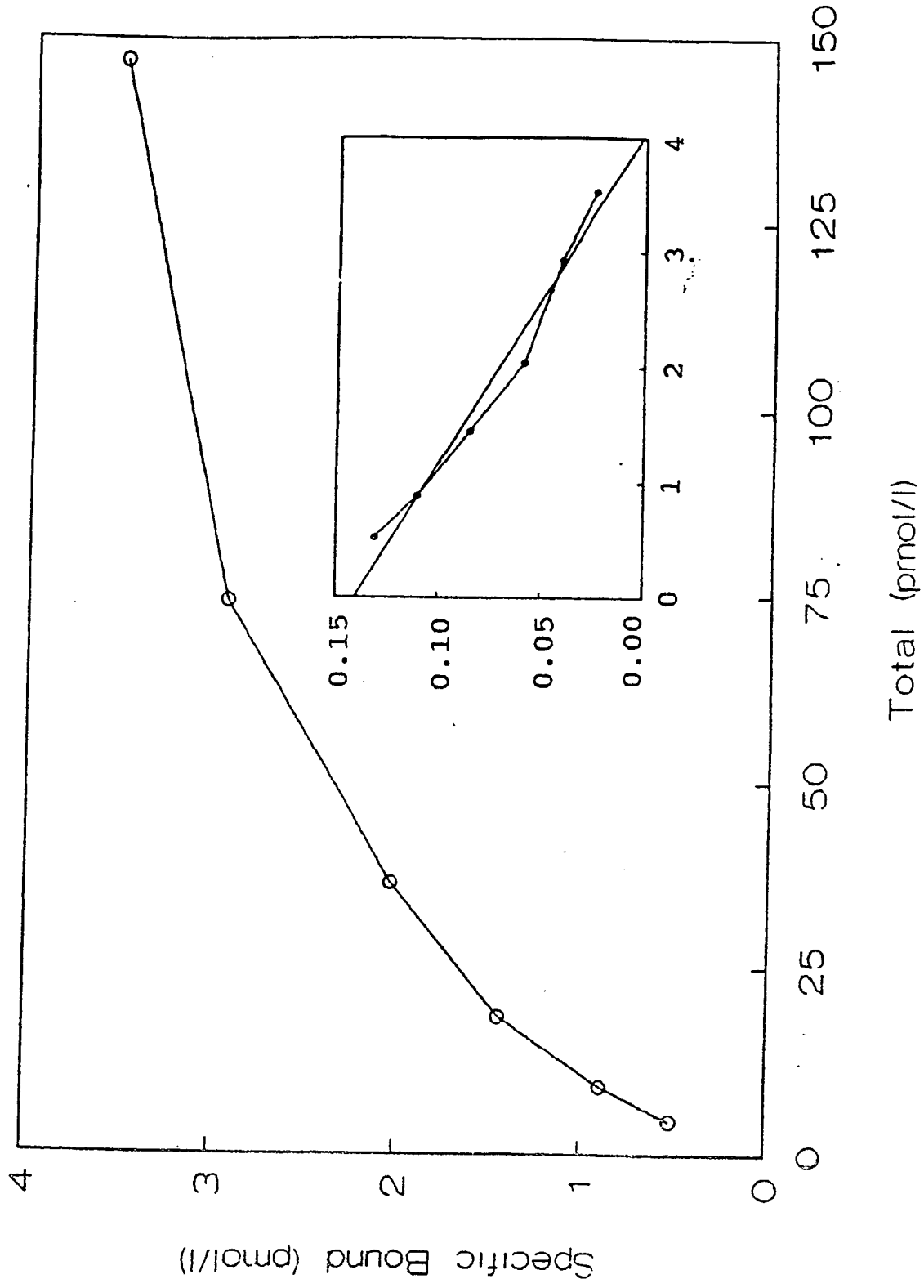

FIG. 3: ^{125}I-hFSH saturation

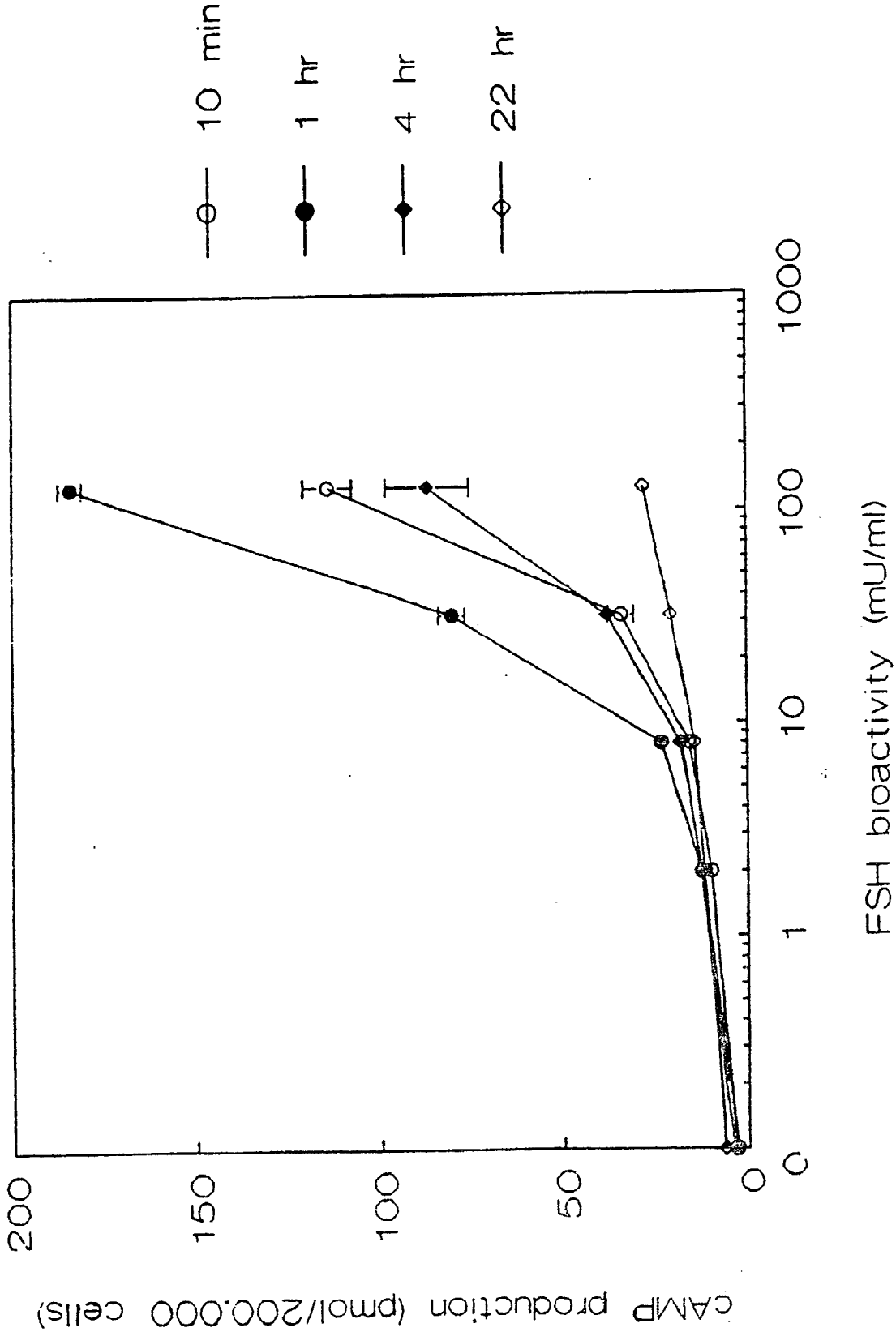

FIG. 4: cAMP production by CHOhFSH-R1Cd10 cells

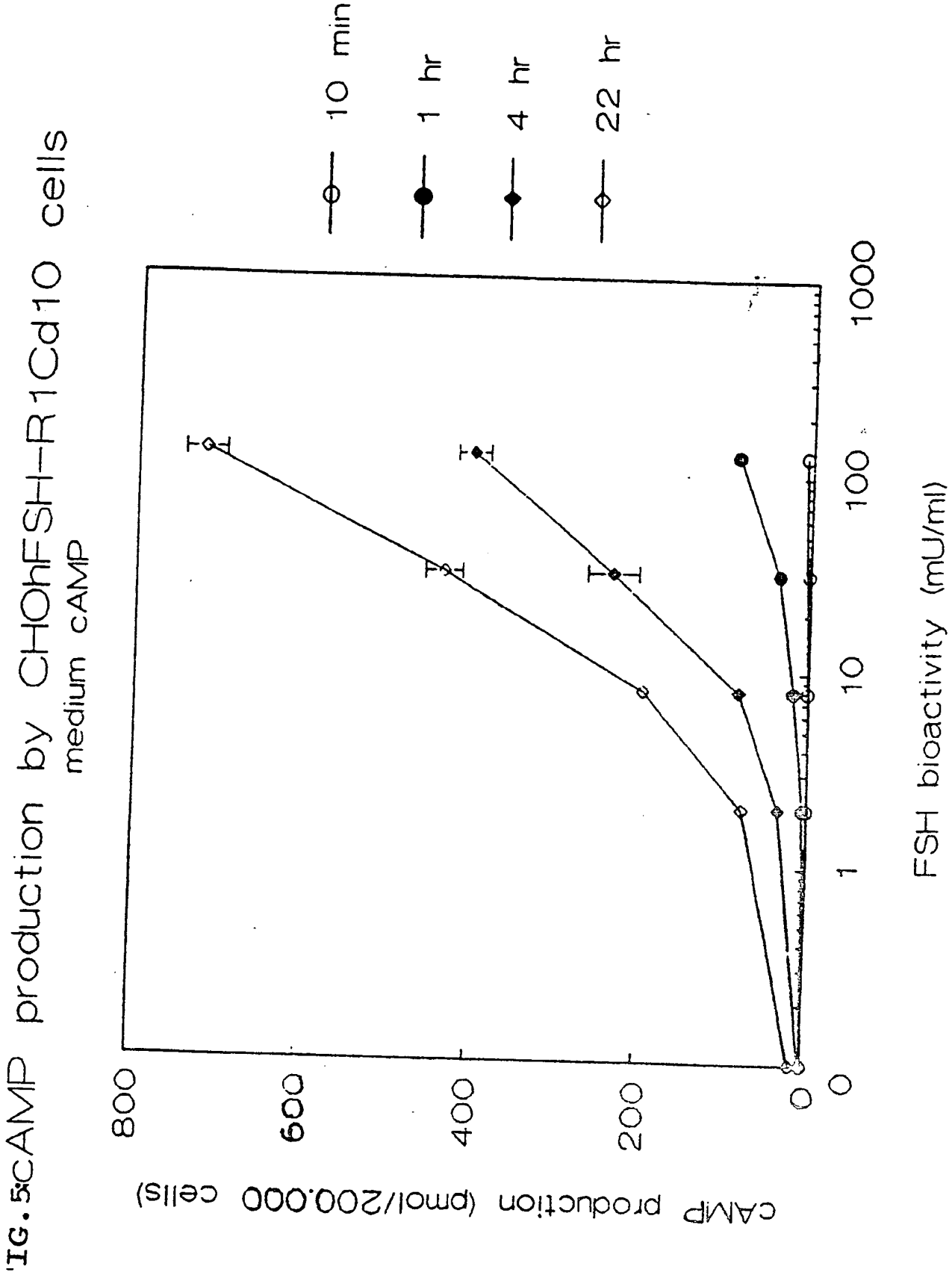
10 min
1 hr
4 hr
22 hr
cAMP production by CHOhFSH-R1Cd10 cells
medium cAMP
cAMP production (pmol/200.000 cells)
0
200
400
600
800
0
1
10
100
1000
FSH bioactivity (mU/ml)

FIG. 5

WESTERN-BLOT OF GST-hFSH-R FUSION PROTEINS STAINED WITH ANTISERA RAISED AGAINST GST-hFSH-R1 AND GST-hFSH-R3

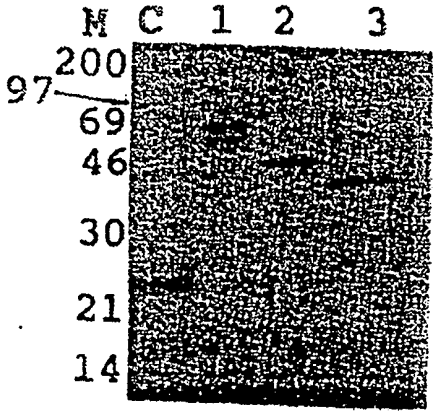

FIG.6A
Coomassie stained

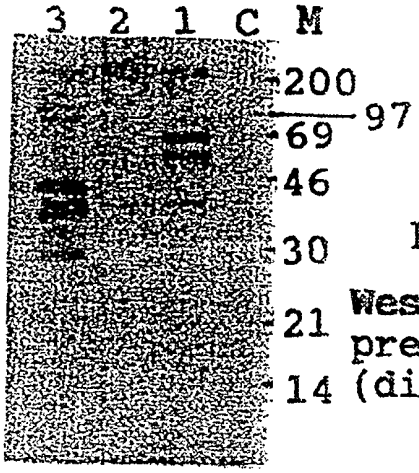

FIG.6B
Western immunoblot with precleared anti GST-hFSH-R3 (dilution 1:8000)

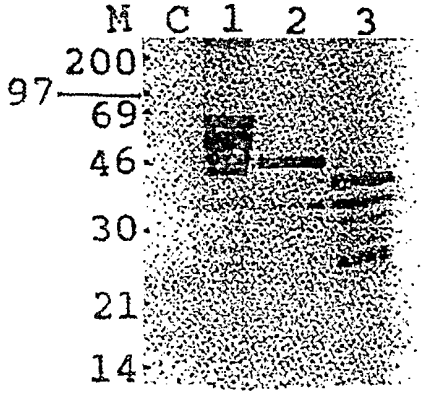

FIG.6C
Western immunoblot with anti GST-hFSH-R1 (dilution 1:250)

M = marker in kD
C = controle (GST)
1 = GST-hFSH-R1
2 = GST-hFSH-R2
3 = GST-hFSH-R3

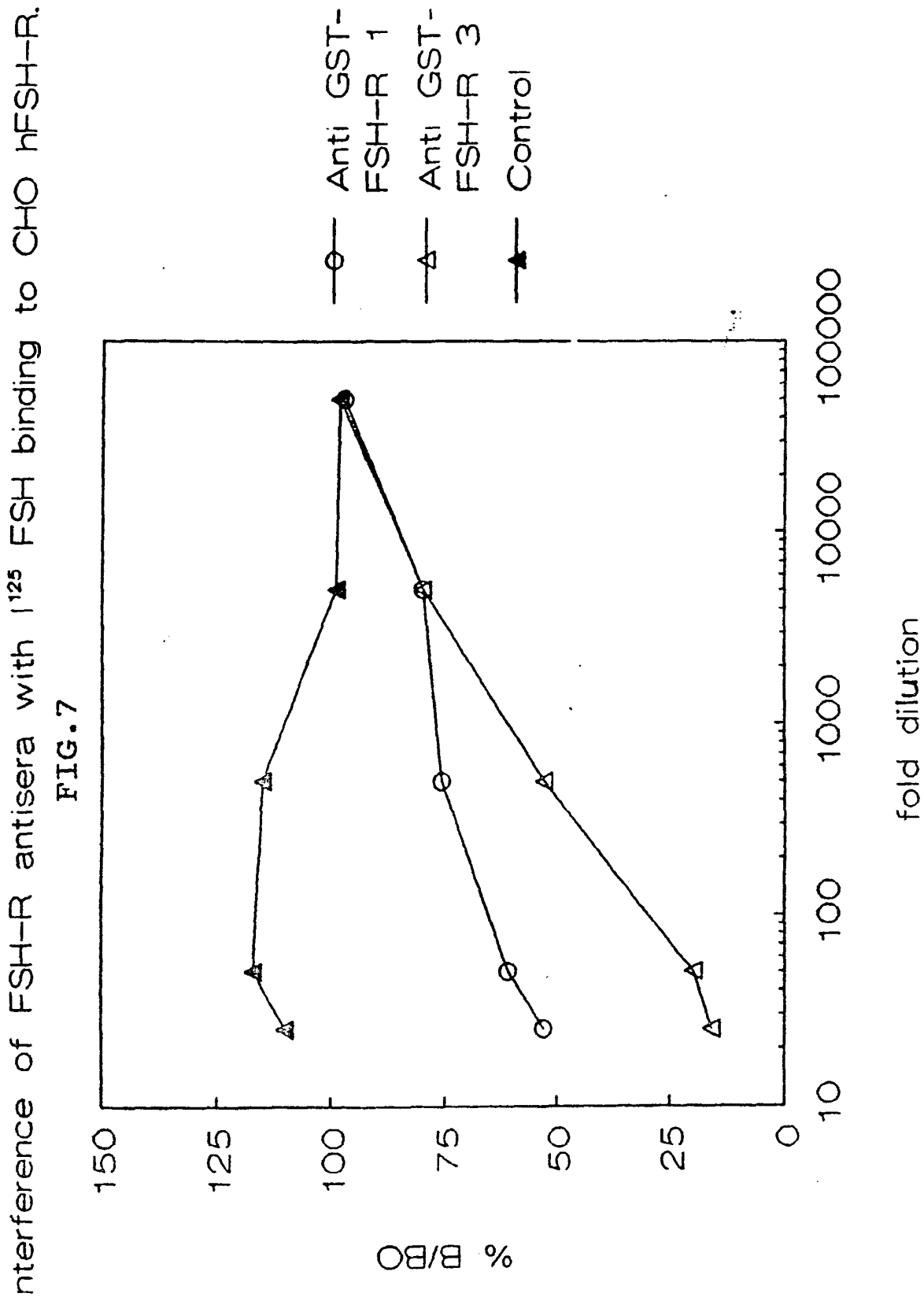
Interference of FSH-R antisera with I^{125} FSH binding to CHO hFSH-R.
Anti GST-FSH-R 1
Anti GST-FSH-R 3
Control
% B/BO
150
125
100
75
50
25
0
10
100
1000
10000
100000
fold dilution

FIG.7

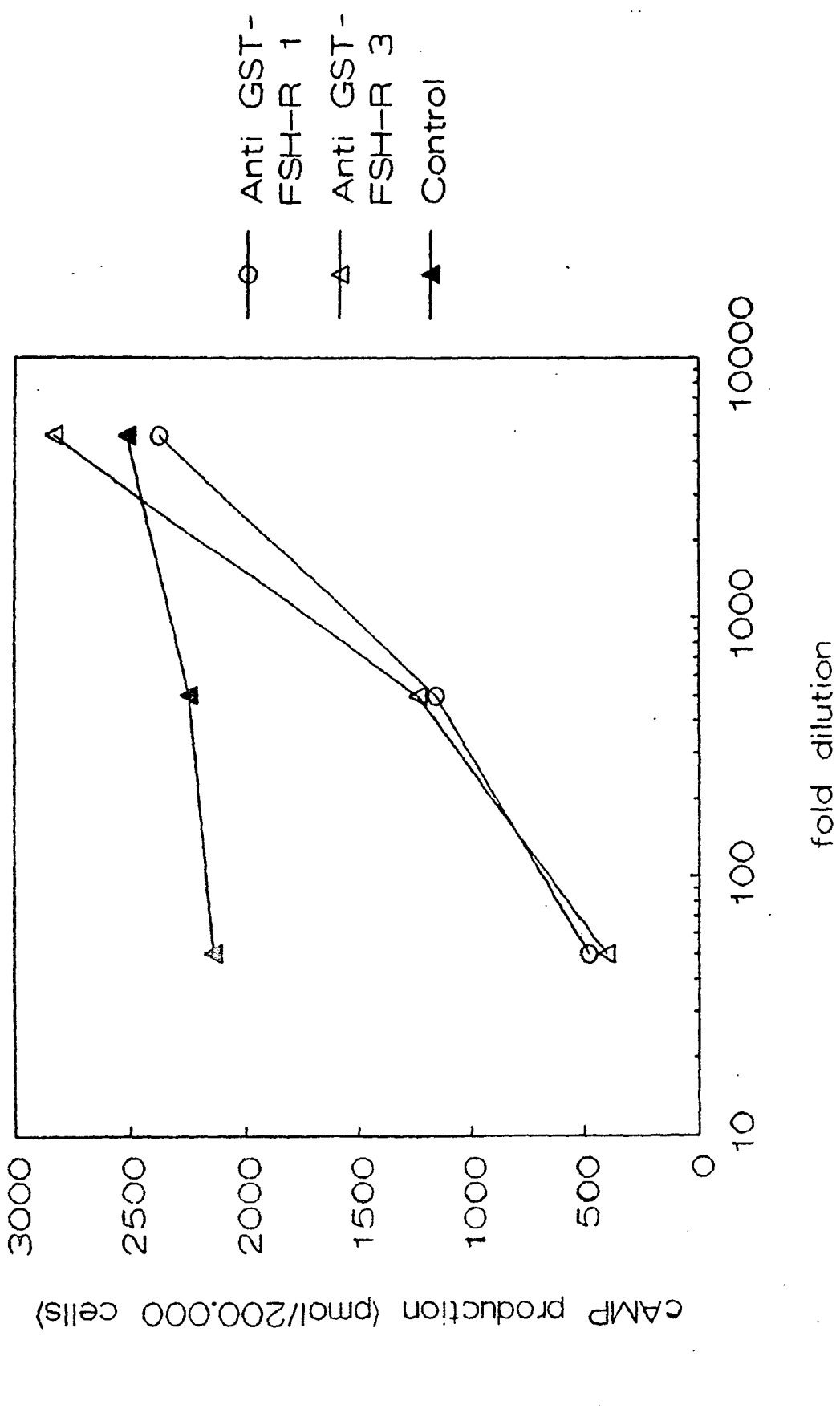

FIG. 8: Interference of FSH-R antisera with cAMP generation.

I. CLASSIFICATION OF SUBJECT MAT[redacted] (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C12N5/10; C12P21/08; C07K13/00
G01N33/53; G01N33/563

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. vol. 175, no. 3, 29 March 1991, DULUTH, MINNESOTA US pages 1125 - 1130 Minegish, Takashi; Nakamura, Kazuto; Takakura, Yumi; Ibuki, Yoshito; Igarashi, Masao 'Cloning and sequencing of human FSH receptor cDNA'	6-17
Y	see the whole document	18-20

Y	US,A,4 921 808 (SCHNEYER, ALAN L. ET AL.) 1 May 1990 see page 3, line 5-62; claims 1-9; example 8	18-20

	-/--	

[10] Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 JULY 1993	30.07.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NAUCHE S.A.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. EP 9300780
SA 72310

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 14/07/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4921808	01-05-90	None		
WO-A-9013643	15-11-90	AU-A-	5732190	29-11-90
		EP-A-	0471030	19-02-92
		JP-T-	4505103	10-09-92
WO-A-8808719	17-11-88	AU-A-	1962788	06-12-88
US-A-4652450	24-03-87	None		
WO-A-9216620	01-10-92	AU-A-	1251492	21-10-92